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Fluorescence Detected Circular Dichroism

Sir:

Circular dichroism (CD) is a powerful technique for investigating molecular structure. The local structure near a chromophore in a macromolecule can be studied, if the CD of the chromophore can be isolated. We report here a new method, fluorescence detected circular dichroism (FDCD), which is capable of measuring the CD spectrum of a single chromophore in a macromolecule, provided the chromophore is fluorescent. Preliminary experimental results are given which show good agreement with theory for simple model systems.

The method is based on the usually valid assumption that the excitation spectrum of a fluorescent chromophore parallels its absorption spectrum.[1] This is generally true for systems in which energy transfer is negligible. In FDCD, the difference in absorption for left and right circularly polarized light is obtained by measuring the difference in fluorescence intensity for left and right circularly polarized excitation.[2] A schematic of the experimental arrangement is shown in Figure 1. The detection system of a Cary 60 spectropolarimeter with Model 6001 CD attachment is modified by placing a 5-cm diameter Hamamatsu R375 photomultiplier at 90° to the excitation beam. This photomultiplier is followed by a preamplifier and the resultant signal is processed by the Cary 60 electronics and displayed on the recorder chart. The pen position is digitized and smoothed in a manner described previously.[4] A colored glass filter is used to discriminate against scattered excitation light, and a standard quartz fluorescence cell is employed. In order to collect more fluorescence, the wall of the cell holder opposite the photomultiplier is made highly reflecting. Baselines are recorded by placing an optically inactive, fluorescent solution in the cell. For compounds with a large FDCD, sodium fluorescein proved adequate. However, for compounds with a small FDCD it is necessary for the baseline solution to approximate the fluorescent characteristics of the solution being measured. This is because the baseline seems to shift a small amount for different fluorescent species. The reasons for this

(1) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **54**, 640 (1958).
(2) Note that the polarization of the fluorescent light is not measured. This is a different method which gives information about the excited state of the chromophore.[3]
(3) J. Schlessinger and I. Z. Steinberg, *Proc. Nat. Acad. Sci. U. S.*, **69**, 769 (1972).
(4) A. D. Blum, O. C. Uhlenbeck, and I. Tinoco, Jr., *Biochemistry*, **11**, 3248 (1972).

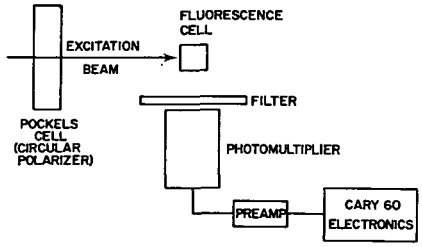

Figure 1. Experimental set-up for measuring FDCD.

shift are not yet understood, but thus far it has been a minor inconvenience.

The spectrometer measures $S = (F_R - F_L)/(F_R + F_L)$ as a function of excitation wavelength, where F_R and F_L are the fluorescence emission intensities for right and left circularly polarized exciting light, respectively. If one assumes that the quantum yield for fluorescence is independent of the wavelength and polarization of the exciting light, then for a solution containing only an optically active, fluorescent species, the CD is related to the FDCD by

$$\epsilon_L - \epsilon_R = \frac{-S(1 - 10^{-A})}{cd10^{-A}} \quad (1)$$

where ϵ_L and ϵ_R are the extinction coefficients for left and right circularly polarized light, A is the absorbance due to the optically active molecule, c is the concentration, d is the pathlength, and S is the measured value of the FDCD, which is equal to the ellipticity reading from the chart divided by 33.0 to give the correct units for $\epsilon_L - \epsilon_R$. This equation was tested by measuring the FDCD of morphine, *d*-10-camphor sulfonic acid, and L-tryptophan. Excellent agreement was obtained in each case. This agreement is strong evidence for the validity of our assumptions in these systems. The results for *d*-10-camphor sulfonic acid are shown in Figure 2. For this case, the absolute magnitude of $\epsilon_L - \epsilon_R$ at the 290 nm peak derived from the FDCD agrees with the previously published value to within 2%.[5]

The extraction of the CD spectrum from the FDCD spectrum is considerably more complicated when optically active, nonfluorescent species are present in addition to the fluorescent chromophore. Application of Beer's law results in the following equation for the CD of the fluorescent species

$$\epsilon_{FL} - \epsilon_{FR} = 2\epsilon_F\left(1 - \frac{2}{1 - \frac{A_L(S-1)(1-10^{-A_R})}{A_R(S+1)(1-10^{-A_L})}}\right) \quad (2)$$

where ϵ_{FL}, ϵ_{FR}, and ϵ_F are the extinction coefficients of the fluorescent chromophore for left circularly polarized, right circularly polarized, and unpolarized light, respectively; A_L and A_R are the absorbances for left and right circularly polarized light due to optically active material. The latter are obtained by measuring both the absorption and CD spectrum for the solution. This equation was verified by applying it to the FDCD spectra of mixtures containing approximately 10^{-5} *M* L-tryptophan and varying amounts of L-cystine in 0.025 *M* sodium phosphate buffer at pH 11. Under these conditions L-cystine is not fluorescent whereas L-tryptophan has a quantum yield of about 50%.[6] At the concentrations used, the CD of the mixture is reproduced by the sum of the CD of its components implying little or no interaction between the L-tryptophan and L-cystine. By measuring the FDCD, CD, and absorption spectra of the mixture, along with the absorption spectrum of L-tryptophan, it should be possible to extract the CD spectrum of L-tryptophan in the mixture. The FDCD baseline for this experiment was provided by scanning a solution of 1.37×10^{-5} *M* DL-tryptophan. To test for artifacts, solutions with ten times the concentration of DL-tryptophan and mixtures of 1.37×10^{-5} *M* DL-tryptophan plus up to 9.95×10^{-5} *M* DL-cystine were also run. All these baselines were essentially coincident. Figure 3 shows a typical result derived from the FDCD of the active mixture. As predicted for little interaction between the components, the CD curve calculated from eq 2 is

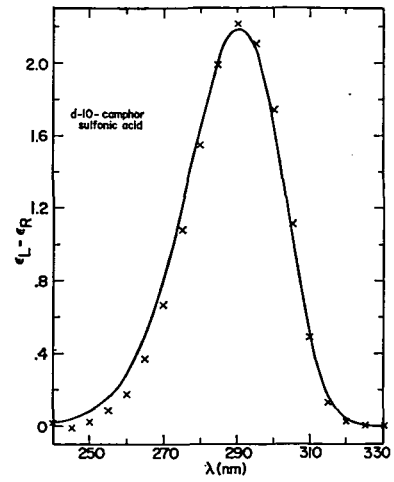

Figure 2. CD spectrum of *d*-10-camphor sulfonic acid: (——) CD spectrum, (×) points derived from FDCD and absorption spectrum using eq 1. Solution measured was 4.30×10^{-3} *M*. Baseline for FDCD was 2×10^{-5} *M* sodium fluorescein. Filter was Corning 4-72. Fluorescence cell had a 1-cm pathlength.

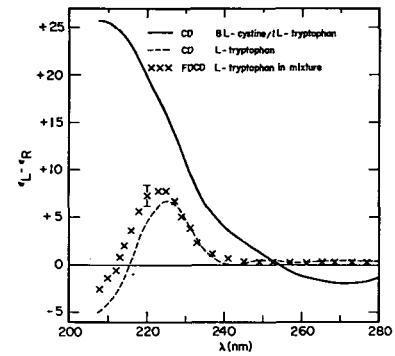

Figure 3. (----) CD spectrum of L-tryptophan, pH 11. (——) CD spectrum of 1.37×10^{-5} *M* L-tryptophan plus 1.157×10^{-4} *M* L-cystine, pH 11, per mole of L-tryptophan. (×) Points derived from FDCD, CD, and absorption spectra of 1.37×10^{-5} *M* L-tryptophan plus 1.157×10^{-4} *M* L-cystine using eq 2. Baseline used for FDCD was 1.37×10^{-5} *M* DL-tryptophan, 0.025 *M* sodium phosphate, pH 11. Filter was Schott WG 295–2 mm. Fluorescence cell had a 0.3-cm pathlength. The error bar shows noise amplitude prior to final smoothing. Similar results were obtained with mixtures containing 1.37×10^{-5} *M* L-tryptophan plus 5.79×10^{-5} *M* L-cystine and 1.08×10^{-6} *M* L-tryptophan plus 5.4×10^{-6} *M* L-cystine.

(5) J. Y. Cassim and J. T. Yang, *Biochemistry*, **8**, 1947 (1969).

(6) R. W. Cowgill, *Biochem. Biophys. Acta*, **75**, 272 (1963).

very close to the CD curve for pure L-tryptophan. For comparison, the CD for the entire mixture is also shown.

These encouraging results on mixtures have led us to extend our experiments to macromolecules. Thus far we have measured the FDCD spectra of lysozyme and t-RNA^{Phe}. Here the fluorescent chromophores are the tryptophans and the anticodon loop Y base, respectively.[7,8] The signal-to-noise ratios in both spectra are several times larger than for the mixtures reported above. This is reasonable since the CD of free L-tryptophan is rather small relative to the CD of a residue in a protein or nucleic acid. To analyze these data, it will be necessary to obtain a value of ϵ_F for the chromophore in its macromolecular environment. There are several possible ways to accomplish this, including measurement of the corrected excitation spectra of the solutions.

The FDCD technique makes it possible to measure the CD of only the fluorescent chromophores in a macromolecule, provided there is little or no energy transfer. This greatly enhanced specificity for CD measurements is very difficult, if not impossible, to obtain by other methods.[9] Unique structural information on selected regions of biologically important macromolecules may thus be obtained.

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(7) S. S. Lehrer and G. D. Fasman, *J. Biol. Chem.*, **242**, 4644 (1967).
(8) U. L. Raj Bhandary, S. H. Chang, A. Stuart, R. D. Faulkner, R. M. Hoskinson, and H. G. Khorana, *Proc. Nat. Acad. Sci. U. S.*, 57, 751 (1967).
(9) S. Beychok, *Annu. Rev. Biochem.*, 37, 437 (1968).

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